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FINAL REPORT

GENETICALLY ENGINEERING FIELD PEAS FOR ENHANCED FUNGAL RESISTANCE

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Genetically Engineering Field Peas for Enhanced Fungal Resistance (1996-1999)

Final Report
December 15, 1999

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(a) Summary:
The objective of this project was to use genetic engineering to produce pea plants with a greater tolerance to fungal pathogens such as *Mycosphaerella pinodes*, for which there is no effective, natural resistance. To accomplish this, genes coding for proteins capable of inhibiting the growth of fungi (antifungal proteins) were introduced into the genome of pea plants where they were expressed at high levels prior to infection. When tested under controlled conditions, leaves from plants expressing the antifungal protein "chitinase", which degrades a structural component of fungal cell walls, or "defensin", which makes holes in the fungal cell membrane, displayed reduced disease symptoms compared to non-transformed pea lines. Leaves from plants expressing a combination of chitinase and another protein capable of degrading a cell wall component (β-glucanase) displayed less disease symptoms than the line expressing only the chitinase. These results indicate that expressing high levels of antifungal proteins may be an effective strategy for reducing fungal diseases in peas. We now need to test the transgenic lines at the whole-plant level and, if possible, under field conditions. Some of these tests are currently being initiated in collaboration with plant pathologists.

(b) Technical Report:

BACKGROUND

Fungal diseases can greatly reduce the profitability of legume production, through their effects on yield, seed quality and costs of chemical control. Despite extensive screening of germplasm, there remains no effective source of resistance to important field pea diseases such as Mycosphaerella blight and foot rot (*Mycosphaerella pinodes, Ascochyta pinodella*), and Fusarium root rot (*Fusarium solani)*. Therefore, we are exploiting biotechnology as an alternative to conventional breeding for solving this problem.

In 1995, the Legume Biotechnology Group at the Plant Biotechnology Institute was able to demonstrate introduction of functional foreign genes into pea. This ability to transform peas opened the door to a wide range of new research approaches that could be used for legume improvement. There was good evidence from work in canola and tobacco that plants genetically engineered to accumulate high levels of proteins capable of inhibiting fungal growth (antifungal proteins) have enhanced resistance to fungal pathogens (Jach et al., 1995; Terras et al., 1995; Zhu et al, 1994). The reduction of disease symptoms achieved using this strategy was impressive (60-75 %), albeit results were only available from tests performed under controlled laboratory conditions. With PBI's recently developed capabilities for inserting foreign genes into peas, it became feasible to produce peas with similar properties, in an attempt to help reduce fungal diseases.

Therefore, the objectives of the project, as detailed in the research proposal were:

1. To procure genes coding for different antifungal proteins and fuse these to gene regulatory elements that would allow them to be expressed at high levels throughout the transgenic plants, even in the absence of infection. These gene fusions would be located in specialized T-DNA plasmid vectors to allow their delivery into pea cells by *Agrobacterium tumefaciens*-mediated genetic transformation.
2. To test the efficacy of antifungal proteins on pea pathogens using an *in vitro* assay.
3. To produce transgenic peas containing individual antifungal protein genes and screen different transgenic lines to identify those expressing the genes at the highest levels.
4. To obtain homozygous, true-breeding lines of transgenic plants.
5. To use cross-pollination to generate lines containing combinations of antifungal proteins.
6. To test the transgenic plants for resistance to infection by *M. pinodes*.

The following sections detail our activities and achievements for each of these objectives.

RESULTS

1a. Gene Isolation:

Although several classes of antifungal proteins have been described in plants (Bowles, 1990; Lamb et al., 1992; Collinge et al., 1993; Bohlmann, 1994; Broekaert et al., 1995), the resources available and the low efficiency of pea transformation necessitated that we restrict ourselves to 2 or 3 genes. We decided to pursue three classes of antifungal proteins which appeared to be the most effective based on previous studies in transgenic plants: chitinases, 1,3-ß-glucanase and defensin peptides. Also, in an attempt to produce transgenic peas as soon as possible, we sought to obtain transformation vectors containing antifungal proteins directly from colleagues. This strategy would allow us to by-pass the time-consuming gene isolation and vector construction steps. Unfortunately, such transformation vectors were either unavailable or were not effective in peas (see objective #3). Consequently, a certain amount of genetic engineering was required with all classes of antifungal protein genes.

(i) Defensins:

Defensins are small cationic peptides that produce pores in the membranes of fungi, resulting in their death (Broekaert et al., 1995; Hancock and Lehrer, 1998). In plants, the best-characterized defensins are those from radish, which have been shown to increase tolerance to fungal pathogens when overexpressed in tobacco plants (Terras et al., 1995). We sought to obtain the radish AFP2 gene from Zeneca Inc., however, problems arose in negotiating a Material Transfer Agreement (MTA) which made their acquisition impractical. Instead, we isolated the AFP2 gene by the polymerase chain reaction (PCR; Ausubel et al., 1989).

In addition to the radish defensins, two pathogen-induced genes from peas showing sequence similarity to defensins had been reported at the time the project was initiated (Chiang and Hagwiger, 1991). We tried to isolate these genes by PCR amplification as well. We succeeded in isolating the defensin gene called PI230a, but did not obtain PI230b (also known as PI39). Instead, we isolated a new member of the family, which we have called PI230c. The sequences of the pea and radish defensins are presented in Figure 1. In order to facilitate future cloning steps, restriction enzyme recognition sites were included in the oligonucleotides used for PCR.

(ii) Chitinases:

Chitinases are enzymes that break-down chitin, a major carbohydrate found in the cell wall of fungi (Collinge et al., 1993). Two chitinase genes were obtained through MTAs, one from rice (Zhu et al., 1994) and the other from the biocontrol fungus *Trichoderma harzianum* (Lorito et al., 1998). These genes had previously been shown to enhance disease resistance when overexpressed in tobacco and/or potato.

(iii) Glucanase:

ß-1,3-glucanase, like chitinase, break-down a major carbohydrate found in the cell wall of fungi (in this case ß-1,3-glucans). We obtained a gene from alfalfa through MTA that had been shown to enhance disease resistance when overexpressed in transgenic tobacco (Zhu et al., 1994). Furthermore, this glucanase was reported to interact synergistically with the rice chitinase to enhance disease resistance along when co-expressed in the same plants (Zhu et al., 19994).

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(1)      PI230a MEK-KSLACLSFLLL-VLFVAQEIVVSEANT-CENLAGSYKGVCF--GGCDRHCRTQEGAISGRCRDDF---RCWCTKNC.
(2)      PI230b MEK-KSLAALSFLLLLVLFVAQEIVVTEANT-CEHLADTYRGVCFTNASCDDHCKNKAHLISGTCHD-W---KCFCTQNC.
(3)      PI230c MEK-KSLAALSFLFLF-LFVAQEIVVTEANT-CEHLADTYRGVCFTDASCDDHCKNKAHLISGTCHN-F---KCFCTQNC.
(4)      AFP2   MAKFASIIVLLFVALVVFAAFEEPTMVEAQKLCQRPSGTWSGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC.
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Figure 1. Amino acid sequence of defensin peptides from peas (1-3) and radish (4). Residues that are conserved between all peptides are highlighted. Note the conserved positioning of cysteine (C) residues. Defensin PI230c was isolated in this research program.

1b. Construction of transformation vectors:

The general strategy used to produce transformation vectors is diagrammed in Figure 2. Standard recombinant DNA methods were used (Sambrook et al., 1989; Ausubel et al., 1989). To ensure high levels of gene expression in the transgenic plants the part of the gene that produces the protein (called the coding region) was separated from the part that normally controls its expression (called the promoter). The promoter of the antifungal proteins that we selected allow the expression of the corresponding coding regions only after pathogen challenge. To obtain high levels of expression throughout uninfected, as well as infected plants, the coding region of antifungal protein genes were separately fused to the cauliflower mosaic virus 35S transcript promoter (CaMV 35S p). In fact, we used a tandem repeat of the CaMV 35S promoter and the alfalfa mosaic virus (AMV) translational enhancer (Datla et al., 1993) to obtain even higher levels of gene expression. As detailed later, fusions to the CaMV 35S promoter were not always effective, and consequently, some of the antifungal protein gene coding regions were also fused to a light-inducible promoter from the pea ribulose bisphosphate carboxylase/oxigenase small subunit gene (rbcS3A) (Kuhlemeier et al., 1987). The promoters were fused to the beginning of the coding regions. To obtain proper expression in plants, we also had to fuse appropriate regulatory elements at the end of the coding regions. For this purpose we used sequences originating from the nopaline synthase gene (Bevan et al., 1983). The *Trichoderma* chitinase gene that we obtained by MTA had already been fused to the double CaMV 35S promoter – AMV enhancer and the nopaline synthase terminator.

Once the coding regions were fused to appropriate regulatory elements, they were incorporated into T-DNA transformation vectors. Such vectors possess T-DNA border repeats required for transfer from bacteria to plants, as well as a selectable/screenable marker gene that allows the recovery of transgenic shoots. The transformation vectors used in this study were pBI3008 and its derivative, pBI3010 (Polowick et al., 2000). These vectors were constructed by PBI's Legume Biotechnology Group, and shown to be effective for the production of transgenic peas. Diagrams showing the relevant features of pBI3008 and pBI3010 are shown in Figure 3. Diagrams of the T-DNA portions of the transformation vectors constructed and used in this study are shown in Figure 4. Note that there are several possible arrangements of the antifungal protein genes and the selectable marker gene within the T-DNA vectors. We constructed vectors with several of the possible arrangements using the glucanase fusions. Results obtained with these vectors were used to construct the most effective arrangements for defensins and chitinase fusions.

Note that all of the manipulations up to this point were performed in the common laboratory bacteria *Escherichia coli*, because it is easy to work with. Once the transformation vectors containing the antifungal genes were completed, they were transferred to the *Agrobacterium tumefaciens* strain EHA105, which is routinely used for pea transformation at the PBI (Polowick et al., 2000).

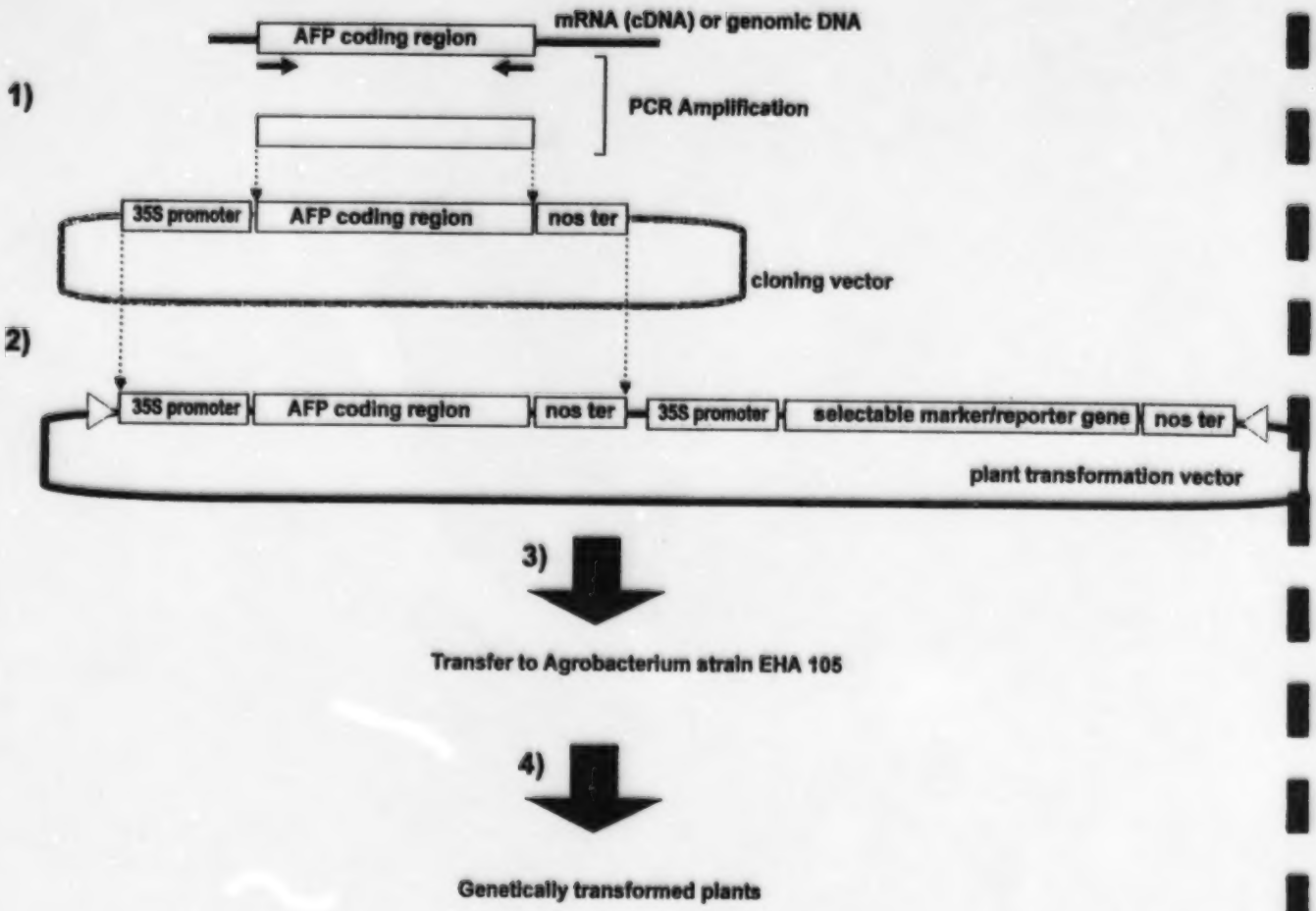


Figure 2. General strategy for cloning Antifungal Protein (AFP) Genes and Construction of Transformation Vectors.

Step 1. Oligonucleotide primers specific to either ends of the coding region portions of the genes were synthesized and used to amplify these regions by the polymerase chain reaction. To ensure high levels of expression throughout transgenic plants, the resulting fragments were inserted between the CaMV 35S promoter and nopaline synthase terminator in a standard cloning vector.

Step 2. The chimeric 35Spromoter-APF coding region-nos terminator genes were inserted into an appropriate plant transformation vector (pBI3010) which also contained a gene conferring resistance to the antibiotic kanamycin for selection of transformed shoots, and a reporter gene (called GUS) to enable rapid identification of transformed shoots. Triangles represent T-DNA border sequences required for transfer of DNA from *Agrobacterium* to plant cells.

Step 3. Desired transformation vectors containing the AFP genes under the control of the 35S promoter are transferred to *Agrobacterium strain* EHA105.

Step 4. Plant tissues are dipped in a suspension the *Agrobacterium* strain and co-cultivated for a few days. The tissues are then moved onto medium containing antibiotics to kill the bacterial cells and to select for cells having received the transferred DNA.

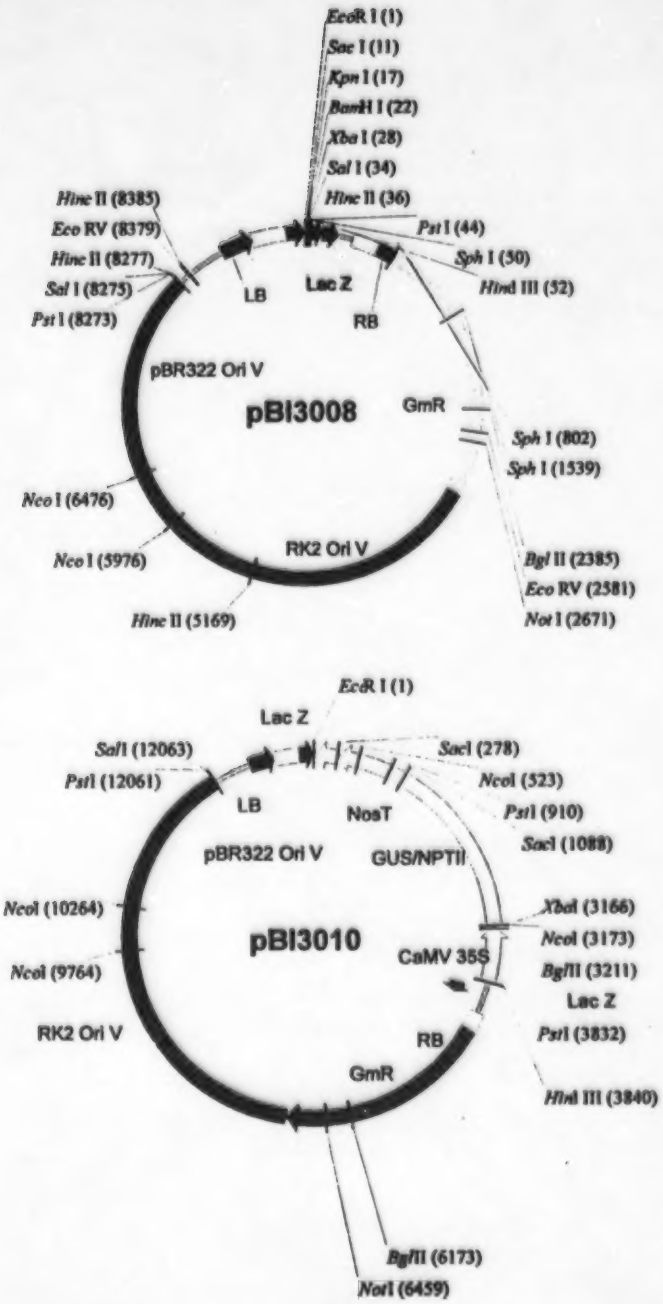


Figure 3. Maps of T-DNA transformation vectors used in this study. LB and RB represent the left and right T-DNA border repeats; pBR322 OriV is a high-copy origin of vegetative plasmid replication in *E. Coli* bacterial cells; RK2 OriV is an origin of plasmid replication in *Agrobacterium* cells; GmR is a gene encoding resistance to the antibiotic gentamycin, for selection in bacterial cells; LacZ is a screenable marker incorporated to facilitate cloning into the multiple cloning site of pBI3008. The name and positions of various restriction enzyme recognition sites are also marked (e.g. NcoI). The meaning of additional terms are defined in the text. pBI3010 is a derivative of pBI3008, containing a tandem CaMV 35S-AMV enhancer - GUS/NPTII - Nos terminator chimaeric selection gene inserted into the EcoRI-HindIII sites.

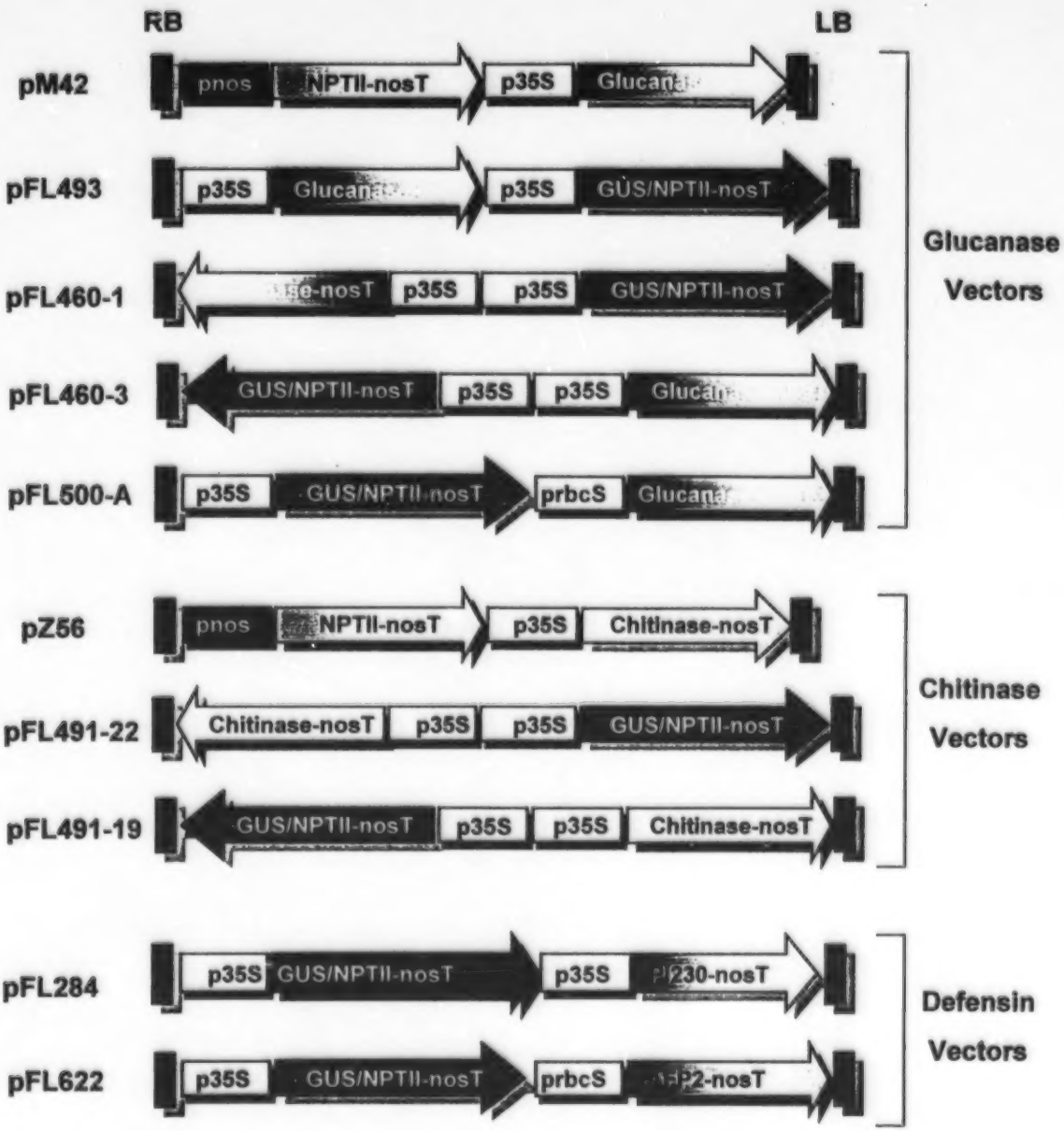


Figure 4: Diagram of T-DNA portion of transformation vectors used in this study.

Note that the relative orientation of the antifungal protein genes and the selectable markers (either GUS/NPTII or NPTII) is variable in these vectors, as is the positioning of the antifungal protein genes relative the right or left (RB, LB, respectively) T-DNA border repeats. Vectors pFL500-A and pFL622 contain the antifungal protein gene coding sequences under the control of the pea rbcS promoter, while all other vectors contain fusions to the CaMV 35S promoter. Vectors pM42 and pZ56 contain the selectable marker under the control of the nopaline synthase (nos) promoter. pM42 and pZ56 are in the binary vector pBIN19, all other vectors are derivatives of pBI3008.

2. Antifungal activity of pea defensins:

Because the pea genes PI230a and PI230c had never actually been shown to possess antifungal activity, we decided to test their ability to inhibit the growth of legume pathogens before proceeding any further.

Because the chemical of synthetic defensins would be expensive, and the production of highly purified defensins expensive and time-consuming, we decided to produce the pea defensins in transgenic tobacco plants. Tobacco was chosen for this purpose because it is much easier to genetically engineer than peas. Transgenic tobacco plants expressing the highest levels of pea defensins were identified by northern blot hybridization. These plants were used to prepare tissue extracts that were tested using a microtitre plate bioassay (Broekaert et al., 1990). Table 1 describes the extraction protocol and summarizes the results obtained. Note that several of the fungi tested are the causative agents of economically important pulse diseases. Extracts from plants expressing pea defensins were more effective at inhibiting the growth of fungi than were extracts from untransformed tobacco plants. This effect was shown to be statistically significant in many cases. These data confirm that the vectors used to transform peas (identical to the one tested in tobacco) can, in fact, enhance the antifungal activity of plant tissues. They also emphasize the importance of producing several independent transgenic plants with each foreign gene and identifying plants with high levels of antifungal gene expression, since only the plants with a high level of expression were found to have enhanced antifungal activity.

The expression patterns of the pea defensin genes had not been studied in detail at the time we initiated this study. Because this information could potentially provide valuable information about the role of these proteins in the plant's response to *M. pinodes* infection, their expression patterns were characterized in more detail. Using either the PI230a or PI230c probe, relatively high levels of expressions were observed in mature leaves, tendrils, stems and flowers (Figure 5). Relatively low levels of transcripts were also found in other parts of the plants such as young leaves, roots and pods. To exclude the possibility that gene expression was induced by low-level infection not associated with visible lesions, pea seeds were surface sterilizated and germinated under aseptic conditions on agar-solidified medium. Strong expression of both defensins continued to be observed under these conditions. For comparison, the expression patterns of two pathogen-related protein (PR) genes, PI49 (a PR-10 homolog; Chiang and Hadwiger, 1990) and 1,3-β -glucanase (Chang et al., 1993) were also determined. The pattern of PI49 gene expression was similar to that of the defensins. In contrast, 1,3-β-glucanase expression was barely detectable in mature plants, with the exception of roots. After infection with *M. pinodes*, levels of pea defensins increased in both pods and leaves (Figure 6). Defensin levels were also elevated following infection with compatible, non-compatible and non-host strains of the bacterial pathogen *Pseudomonas syringae*.

3. Production and molecular analysis of transgenic plants:

A summary of our plant transformation activities is presented in Table 2.

(i) Glucanase:

Neither of the two transformation vectors obtained through MTA (pZ56, pM42) were effective at producing transgenic peas. Consequently, we attempted to clone the coding region of the antifungal protein genes from these vectors, fuse them to the tandem CaMV 35S promoter-AMV enhancer (since they were under the control of a single CaMV 35S promoter and no AMV enhancer in pZ56 and pM42) and introduce the new fusion genes into pBI3008 or pBI3010. This

Table 1. Inhibition of fungal growth by protein extracts from transgenic tobacco plants expressing pea PI230a and PI230c transgenes. The total protein extract from leaves was heat-denatured (85°C for 10 min.), and the denatured proteins were removed from the extract by centrifugation. After dialysis of the supernatant, the crude protein preparation was concentrated with a Centricon-3 unit. The desired amount (25 µg/m) of protein from the preparation was applied to the fungal spores in a 1/16 strength of PDB (potato dextrose broth) liquid medium in a well of a microtitre plate. Absorbance readings were obtained after 8 days of incubation at room temperature. The data represent the percentage growth of fungal spore cultures in the presence of plant extracts from an untransformed tobacco plant, and independent transgenic plants containing either PI230a (lines 274) or PI230c (lines 273). Data presented are the average percentage inhibition from four replications, and analyzed with Microsoft Excel data analysis package. The $LSD_{0.05}$ is the least significant differences at 5% significant level.

	Transgenic line							
Fungal Stains	Control	273-1	273-3	273-4	274-3	274-4	274-13	$LSD_{0.05}$
Alternaria alternata	100	81.5	41.3*	50.3*	89.3	99.8	57.2*	30.5
Ascochyta lentis	100	77.9	34.8	26.0	70.3	83.9	57.3	NS
Ascochyta pinodes	100	109.0	44.9*	61.4*	83.8	45.6*	36.1*	25.6
Ascochyta pisi 040	100	82.1	43.6*	46.7*	68.3*	55.7*	38.7*	29.5
Fusarium oxysporum pisi	100	87.3	38.4*	67.1	83.9	109.5	71.6	40.7
Fusarium solani pisi	100	103.8	49.8*	62.3*	95.5	123.0	82.4	31.8
Leptosphaeria maculans	100	112.9	47.2*	89.3	80.9	29.2*	88.6	47.7
Trichoderma reesei	100	55.9*	29.5*	33.2*	65.0*	14.3*	39.1*	35.2

(* = Significantly different from control treatment at 5% level. NS = Not significant at 5% level)

(We a very grateful to Dr. Allen Xue, Agriculture Agri-Food Canada, Mordern Research Station for his kind gift of the fungal isolates used in this study and for supplying methods for their growth and maintenance)

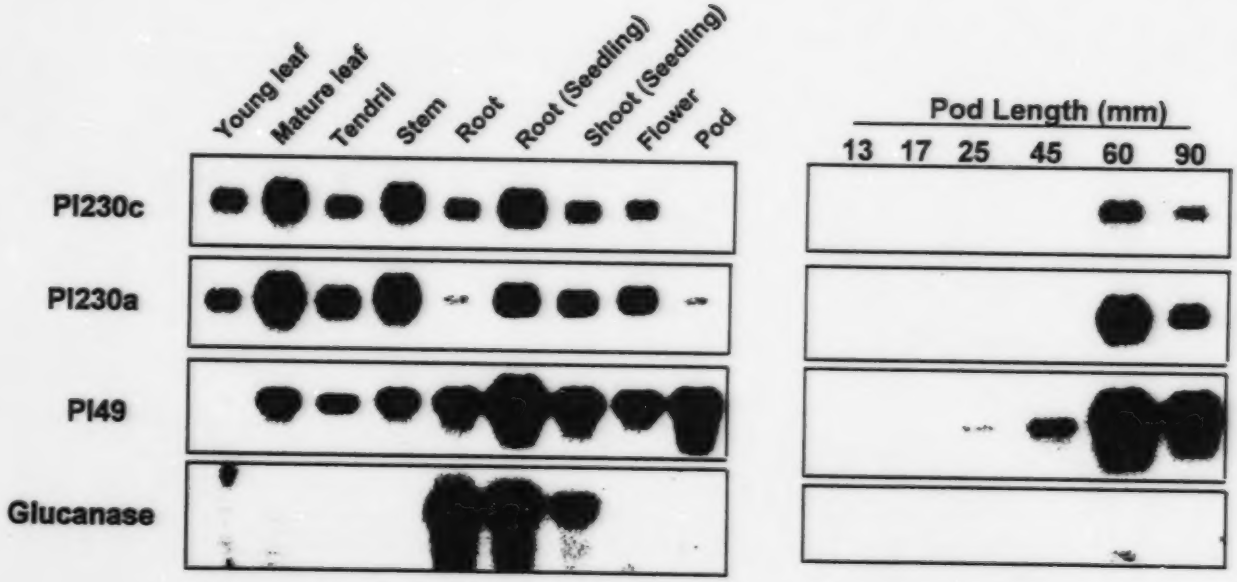


Figure 5. Expression of pea defensin genes in unifected plants.

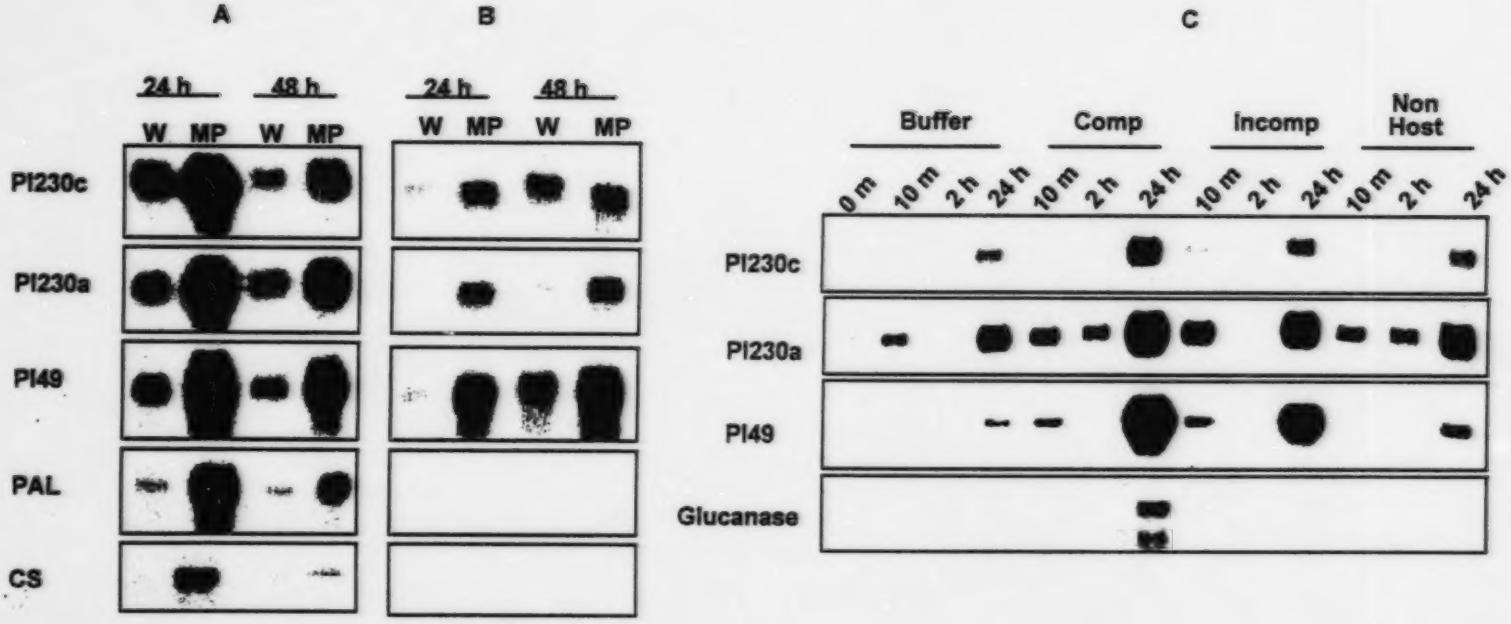


Figure 6. Expression of pea defensins following pathogen challenge.

A) Leaves following challenge with water (W) or *M. pinodes* (MP).
B) Pods following challenge with water (W) or *M. pinodes* (MP).
C) Leaves following challenge with compatible (Comp), Incompatible (Incomp) or non-host strains of *P. syringae*.
In addition to defensins, expression of PI49, phenylalanine amonia lyase (PAL), chalcone synthase (CS) and glucanase was monitored.

Table 2. Summary of Pea Transformation with Antifungal Protein Genes.

Antifungal Protein Gene	Vector Name	Relevant Feature	# Explants	# Shoots	# Plants	# Plants Expressing Genes	# Stong Expressors
Alfalfa glucanase	pFL460-1-1a	Glucanase gene fused to double CaMV 35S promoter; near RB, opposite orientation of selectable marker	1760	4	1	0/1	0
	pFL460-1-3a	Glucanase gene fused to double CaMV 35S promoter; near LB, opposite orientation of selectable marker	2320	38	20	3/20	1
	pFL493-5a	Glucanase gene fused to double CaMV 35S promoter; near RB, same orientation as selectable marker	2404	17	7	0/3	0
	pFL500-A2	Glucanase gene fused to RUBISCO promoter; near LB, same orientation as selectable marker	1148	74	46	21/27	17
	PM42-2a	Glucanase gene fused to CaMV promoter; near LB, same orientation as NPT II gene under control of nos promoter	600	0	0	0	0
		Total Glucanase	**8232**	**133**	**74**	**24/51**	**18**
Trichoderma chitinase	pFL491-22a	Chitinase gene fused to double CaMV 35S promoter; near RB, same orientation as selectable marker	2184	26	10	1/9	1
	pFL491-19b	Chitinase gene fused to double CaMV 35S promoter; near LB, opposite orientation of selectable marker	388	0	0	0	0
Rice Chitinase	pZ56-Ba	Chitinase gene fused to CaMV promoter; near LB, same orientation as NPT II gene under control of nos promoter	600	1	1	0/0	0
		TOTAL CHITINASE	**3172**	**27**	**11**	**1/9**	**1**
Defensins	PFL284-4, -5	Pea defensin fused to double CaMV 35S promoter; near LB, same orientation as selectable marker	916	71	37	0	0
	pFL274-1a	Pea defensin fused to double CaMV 35S promoter; near LB, same orientation as selectable marker	920	36	35	0/9	0
	pFL622-8	Radish AFP2 gene fused to RUBISCO promoter; near LB, same orientation as selectable marker	1632	94 +	94	40/94	5
		TOTAL DEFENSIN	**3468**	**201**	**166**	**40/103**	**5**
		TOTAL	***14872***	***361 +***	***251***	***65/163***	***24***

yielded three different transformation vectors; pFL460-1-1, pFL460-1-3 and pFL493-5. These vectors differ only in the positioning of the glucanase gene relative the right and left T-DNA border repeats and/or the relative orientation of the antifungal protein gene with respect to the selectable marker gene (see Figure 4). These vectors did not perform equally, with most plants being obtained with vector pFL460-1-3, where the glucanase gene is found near the left border repeat, and is in the opposite orientation relative the selectable marker gene. The 20 plants generated with this vector were analyzed for glucanase expression using northern blot hybridization, and only 3 were found to express the transgene. Of these, only 1 plant (called D19) contained high levels of glucanase gene expression. Representative data from northern blot hybridization are presented in Figure 7.

Because of the low percentage of transgenic plants expressing the glucanase gene, we sought to modify our transformation vectors. All of the vectors tested to date contained the glucanase gene fused to the CaMV 35S promoter (see Figure 4). This same promoter was also being used to drive the expression of the reporter gene (GUS-NPTII; see Figure 3). We suspected that one possible reason for the low frequency of expression might be related to the presence of multiple copies of the same genetic element within the T-DNA. Consequently, the glucanase gene was fused to the rbcS promoter, which had previously been shown to drive high levels of gene expression in transgenic shoots. The resulting vector, pFL500-A, proved to be the best in terms of the frequency of plants recovered and the frequency of plants expressing glucanase (Table 2).

(ii) Chitinases:

We were unable to isolate the rice chitinase coding region from vector pZ56. This was due in part to errors in the restriction map that had been supplied as part of the MTA. We therefore decided to pursue our studies with the *Trichoderma* chitinase. The tandem CaMV 35S-AMV chitinase fusion obtained through MTA was introduced into pBI3008 to create two vectors; pFL491-22 and pFL491-19. These vectors are very similar, and differ only in the positioning of the antifungal protein gene relative the T-DNA border repeats (see Figure 4). However, we were only able to recover plants from one of the two vectors. Northern blot hybridization revealed that plants from only a single transformation event (D96) expressed the chitinase gene at a high level. Due to lack of time and resources, we were unable to fuse the chitinase gene to the rbcS promoter and test the effectiveness of such a fusion.

(iii) Defensins:

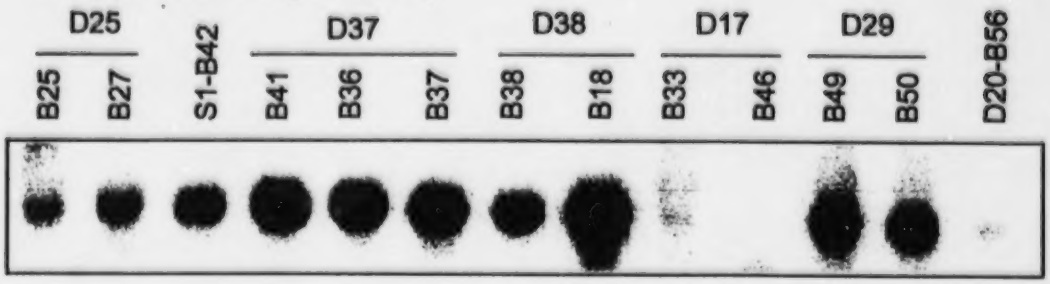
We were unsuccessful at detecting gene expression in any of the plants transformed with pea defensin constructs (pFL284 and pFL274; Table 2). Because pea defensin genes are expressed in leaves of untransformed plants (see Figure 5), we decided to screen young pods for expression of these transgenes (defensins are not naturally expressed in these tissues). By this point in time, we had established that pFL500-A, where the glucanase gene was fused to the rbcS promoter, was highly effective at producing plants which expressed the transgene. Consequently, the radish AFP2 defensin coding region was fused to the rbcS promoter and cloned into pBI3010 to create pFL622-8. As shown in Table 2, this vector proved very effective at producing plants that expressed this defensin gene. In all 5 lines expressing high levels of the radish AFP2 were recovered.

(iv) Summary of transgenic plant production:

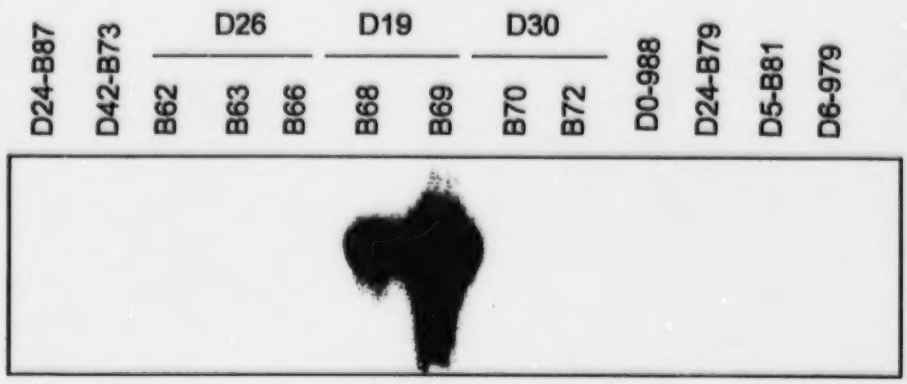
In total, we have generated more than 250 independent primary transgenic plants and identified lines expressing high levels of each of the target antifungal protein genes. Our results indicate that minor changes in the transformation vectors, such as the orientation of the

Alfalfa β- Glucanase

A. rbcS Promoter (pFL500-A)



B. CaMV 35S Promoter (pFL460-1-3)



***Trichoderma* Chitinase CaMV 35S Promoter (pFL491-22a)**

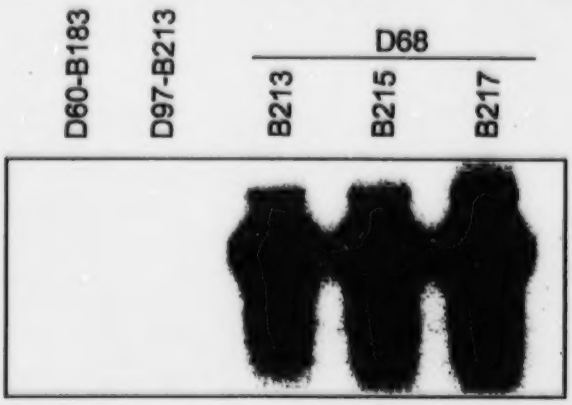


Figure 7. Examples of Northern blot hybridization screening of primary transformants for antifungal protein gene expression. "D" and "S" numbers indicate transformation event, while "B" numbers represent individual plant labels.

antifungal protein gene relative to the marker gene, and the positioning of the genes relative the T-DNA border repeats, can have a significant effect on the number of plants obtained, and the ability of these plants to express the gene of interest at a high level. When both the marker gene and the antifungal protein gene were under the control of the tandem CaMV 35S promoter-AMV enhancer, only a small percentage (5-10%) of the plants expressed the antifungal protein gene at high levels. In contrast, many of the plants (50 – 90%) containing the antifungal protein genes under the control of the rbcS promoter expressed the transgenes. However, in most cases, the absolute level of expression, as determined by northern blot analysis, was highest in the plants with the CaMV 35S promoter.

4. Production and molecular analysis of homozygous lines:

A total of 120 seeds from 20 independent primary transformants (T_0 generation) expressing high levels of defensin or glucanase, and the one line expressing chitinase were sown, and analyzed by northern or slot blot hybridization to identify T_1 plants that expressed the transgenes.

(i) Chitinase:

As shown in Table 3, expression of the chitinase gene segregated 3:1 (expression/no-expression) in the T_1 generation, suggesting the presence of a single copy of the transgene in this line. To determine which of the T_1 lines were homozygous for the transgene, seeds from 2 T_1 lines (B216 and B217) were sown and analyzed in turn for gene expression. All of the T_2 plants analyzed from these T_1 lines expressed the chitinase gene, indicating that they are homozygous for the T-DNA. A large amount of seed (T_3 generation) is now available from these plants for disease testing

Although RNA expression is a convenient method of assessing whether the gene is expressed, it does not conclusively tell us that the gene in question is functional. To determine whether the chitinase gene expressed in the T_2 lines produced above was also functional, the plant material was analyzed for chitinase activity using an *in vitro* assay (Boller and Mauch, 1988). As summarized in Table 4, plants expressing the *Trichoderma* chitinase were found to have 2-5 times more activity than untransformed pea plants. This suggests that the gene is indeed functional in these transgenic lines.

(ii) Glucanase:

Plants derived from two of the T_0 plants expressing the CaMV 35S fusion (D19, B67 & B69) segregated for high levels of glucanase expression, consistent with a single gene copy (Table 3). However, the progeny from the third T_0 plant (B78 - assumed to be from the same transgenic event as the other two) expressed very low levels of glucanase transcript. Consequently, further analysis was restricted to line B67.

Seeds from 4 T_1 B67 plants were sown and analyzed for glucanase expression. All lines continued to segregate for expression of glucanase in the T_2 generation, indicating that none are homozygous. This was unexpected based on the results obtained with T_1 material that suggested a single copy insertion. It is possible that the gene is being shut-off in some of the plants or that a technical error occurred during the analysis.

Two lines (D68 and D37) that expressed the rbcS glucanase fusion at a high level in the T_0 generation only expressed at weak levels in the T_1. In addition, all plants from the D37 line expressed glucanase, suggesting the presence of multiple copies of the transgene in the T_0 plant. A third line, D38, also expressed the glucanase gene at low levels. Consequently, lines D37, D38 and D68 are unlikely to be of interest to us and were not further pursued.

Table 3. Analysis of gene expression in T_1 and T_2 generations of pea lines containing glucanase or chitinase transgenes.

Plants	Seeds sown	# Positive Plants	# Negative Plants	# Homo-zygous Lines	# seeds collected
Chitinase					
T_1 lines					
B216	10	7	3	N.A.	N.C.
B217	6	3	3	N.A.	N.C.
Total T_1	16	10	6		
T_2 lines					
B216-7-2	6	6	0	6	119
B216-7-3	6	6	0	6	131
B216-7-4	6	6	0	6	146
B216-7-6	6	6	0	6	159
B217-4-11	6	6	0	6	119
B217-4-12	6	6	0	6	142
Total T_2	36	36	0	36	866
Glucanase					
T_1 lines					
B67	4	2	2	N.A.	N.C.
B69	4	3	1	N.A.	N.C.
B78	6	6 (weak)	0	N.A.	N.C.
D37-B36	8	8 (weak)	0	N.A.	N.C.
D68-B101	6	1	5	N.A.	N.C.
D97-B231	6	4	2	N.A.	N.C.
Total T_1	34	10	24		
T_2 lines					
B67-4-4	6	4	2	0	70
B67-4-6	6	3	3	0	103
B67-4-8	6	5	1	0	115
B67-4-10	6	3	1	0	99
Total	24	15	7	0	404

Notes: N.A. = not applicable; N.C. = not counted.

Table 4. Analysis of Enzymatic activity in transgenic Plants Expressing Antifungal Protein Genes.

a) Chitinase

Plant	RNA expression	Activity
217-4-1	+	51.1
217-4-2	+	32.9
217-4-3	+	37.5
217-4-4	+	24.7
217-4-5	+	49.7
217-4-6	+	55.6
217-4-7	+	33.5
217-4-8	+	49.5
217-4-9	+	44.7
217-4-10	+	49.1
217-5-1	+	16.1
217-5-2	+	20.6
217-5-3	+	35.2
217-5-4	+	31.3
217-5-5	+	36.3
217-5-6	+	36.7

Plant	RNA expression	Activity
217-5-7	+	34.7
217-5-8	+	31.4
217-5-9	+	46.1
217-5-10	+	52.6
217-5-11	+	43.3
217-5-12	+	43.4
217-5-13	+	32.4
217-5-14	+	36.3
217-5-15	+	31.8
217-5-16	+	15.3
217-5-17	+	27.7
217-5-18	+	20.6
217-5-19	+	32.4
217-5-20	+	29.0
Untransf.		8.0
Untransf		11.2

b) Glucanase

Plant	RNA expression	Activity
231-1	+	48

Plant	RNA expression	Activity
Untransf	-	22

One line (D97-B231) containing the rbcS glucanase fusion continued to express the transgene at a high level. Segregation of glucanase expression in these plants was consistent with the presence of a single transgene in the T_0 generation. Seeds from the T1 lines are available, but we have not been able to study the inheritance of the transgene into the next generation. We have, however, analyzed glucanase activity in the B231 line. Using an *in vitro* assay (Kuffmann et al., 1987), we were able to show that this line contained twice the amount of glucanase activity as non-transformed peas (Table 4), suggesting that the glucanase gene is active in these lines.

(iii) Defensins:

Seeds from 15 lines were screened for AFP2 expression in the T_1 generation. Of these, 3 lines: B643, B702 and B741 were identified as strong expressors. Segregation of gene expression was observed in the first of these lines, suggesting that it may contain a single T-DNA insertion. Based on a limited number of samples, we could not detect segregation in the latter two lines, suggesting that they may contain multiple insertions. Because the defensin plants were the very last to be generated, we have not had time to test the segregation of this transgene in the T_2 generation. However, a large number seeds from T_1 plants, many of which may be homozygous, have been collected (ca. 1,300).

5. Production and molecular analysis of plants expressing a combination of antifungal protein genes.

Selected flowers from T_0 or T_1 plants expressing one type of antifungal protein gene were used to pollinate transgenic plants expressing another type. Table 5 summarizes the results obtained, while Figure 8 shows a representative example of a slot blot analysis.

F_2 generation seeds expressing each possible combination of 2 antifungal protein genes: chitinase + glucanase; chitinase + defensin; glucanase + defensin, have been recovered. In the case of the chitinase and glucanase combination, F_3 material is now available. This generation will have to be screened in order to identify homozygous lines expressing both antifungal protein genes.

6. Analysis of transgenic plants for antifungal activity.

(i) Plants containing single antifungal protein genes.

The ability of transgenic pea plants to resist infection by the fungal pathogen *M. pinodes* was assessed using detached leaves from T_2 plants expressing the glucanase or chitinase constructs and from T_1 plants expressing the defensin gene. In order to describe disease symptoms, an disease index was developed (Table 6). Leaves from untransformed plants or from plants that did not express the antifungal protein genes, as determined by RNA hybridization, were used as control material.

Results for the defensin plants are shown in Figure 9. The data represent 16 replicates for the untransformed Greenfeast control, and 84 replicates for transgenic plants that expressed (AFP+) or failed to express (AFP-) the transgene. Experiments were repeated twice (A and B) on the same plant population. Disease symptoms in leaves from plants expressing the defensin gene were less pronounced than those of control plants. This was more apparent in the trial presented in Figure 9B. Reduction of disease symptoms was more apparent on days 5-9.

Results obtained with the glucanase and chitinase genes are presented in Figure 10. We were unable to observe any reduction of disease symptoms in leaves from plants expressing

Table 5. Generation of transgenic plants expressing combinations of antifungal protein genes.

a)F_1 generation

Female Parent	Male Parent	# Crosses	# F_1 Seeds	# F_1 Plants Expressing both transgenes	# F_2 seeds collected
RbcS-glucanase	35S-chitinase	5	15	3	232
35S-glucanase	35S-chitinase	4	25	0	403
35S-chitinase	rbcS-glucanase	1	5	1	73
35S-chitinase	35S-glucanase	10	42	6	339
35S-glucanase	35S-chitinase	8	40	2	260
35S-chitinase	rbcS-defensin	8	29	3	269
35S-glucanase	rbcS-defensin	5	27	0	139
RbcS-defensin	35S-glucanase	3	14	1?	129
RbcS-defensin	35S-chitinase	4	10	2	121
	Total	48	207	18	1965

a)F_2 generation

Female Parent	Male Parent	# F_2 seeds Sowed	# Plants Expressing both transgenes	# F_3 Seeds
RbcS-glucanase	35S-chitinase	48	21	777
35S-chitinase	rbcS-glucanase	30	11	387
Total		78	32	1164

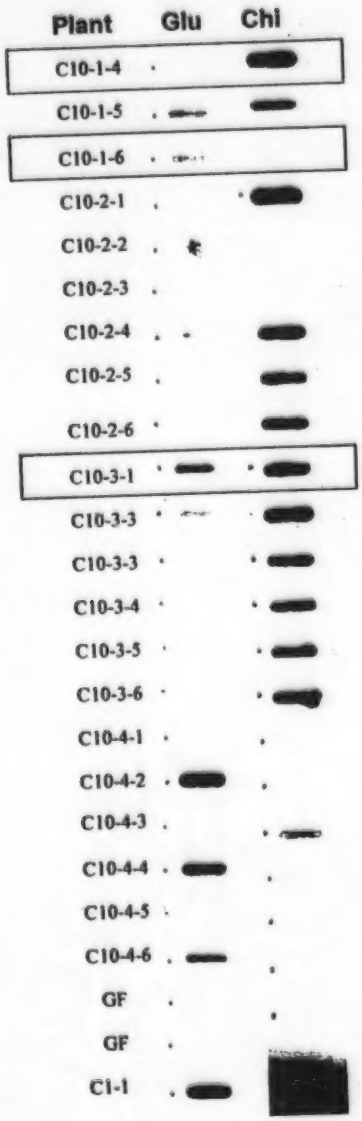
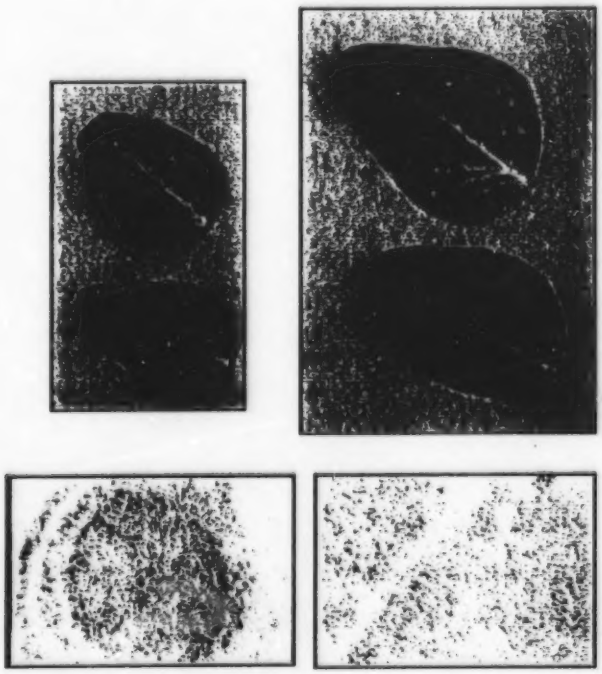


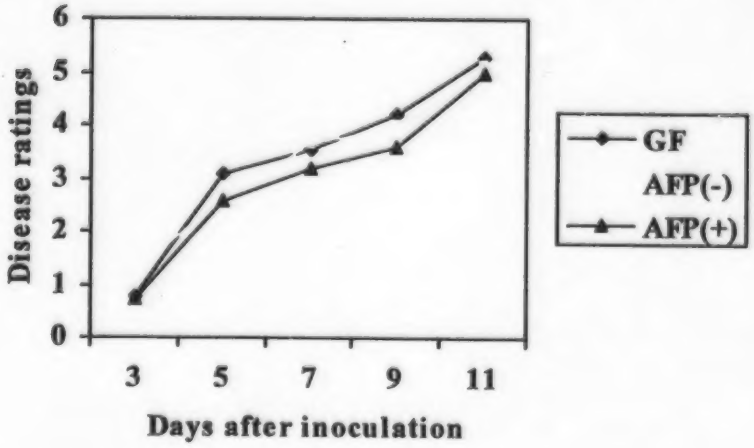
Figure 8. Examples of slot blot hybridization used to identify transgenic plants expressing both the glucanase (Glu) and chitinase (Chi) genes. A plant expressing the chitinase gene only is boxed in red as example. A plant expressing the glucanase gene only is boxed in blue as example. A plant expressing both genes is boxed in black. GF, untransformed Greenfeast controls; C 1-1, control known to contain the antifungal protein genes.

Table 6. Disease symptom index. *M. pinodes* spores (10 µl of $2x10^5$ spores/ml) were inoculated onto detached pea leaves. The inoculated leaves were incubated in a box (15"L X 10"W X 9"H) on top of moist sterile gauze at 100% relative humidity. Disease symptoms on the inoculated pea leaves were evaluated with a light dissection microscope after different times. Photos of typical results from untransformed plants (left) and plants expressing antifungal protein genes (right) are show as well.

Disease index	Description
0	No visible symptom in the inoculated area under microscope
1	10% or less inoculated area shown necrotic lesion or flecks
2	10-30% inoculated area covered with necrotic lesion or flecks
3	30-50% inoculated area covered with necrotic lesion or flecks
4	50-80% inoculated area covered with necrotic lesion or flecks
5	Larger than 80% of inoculated area covered with necrotic flecks or lesion, the lesion becoming confluent, visible mycelium growth into adjacent area
6	Total inoculated area symptomatic and further develops into adjacent area as concentrical rings. Visible fungal growth and adjacent area shown senescence



A



B

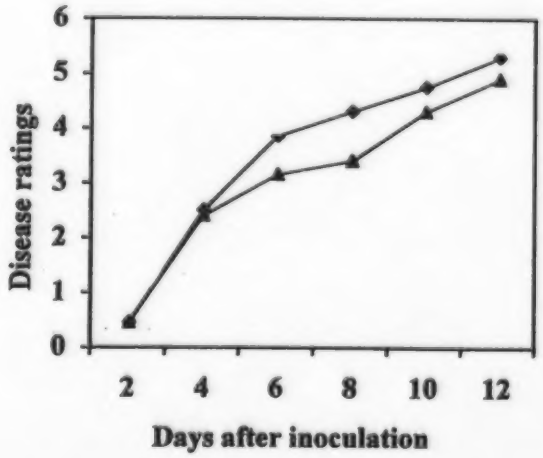


Figure 9. Effect of defensin gene expression on disease symptoms of detached leaves following infection with M. pinodes spores. Disease index used in this study is detailed Table 6. GF, untransformed Greenfeast leaves; AFP+, Greenfeast transgenics expressing the CaMV35Sp-radish AFP2 gene from pFL622; AFP-, transgenci leaves in which AFP2 expression was undetectable.

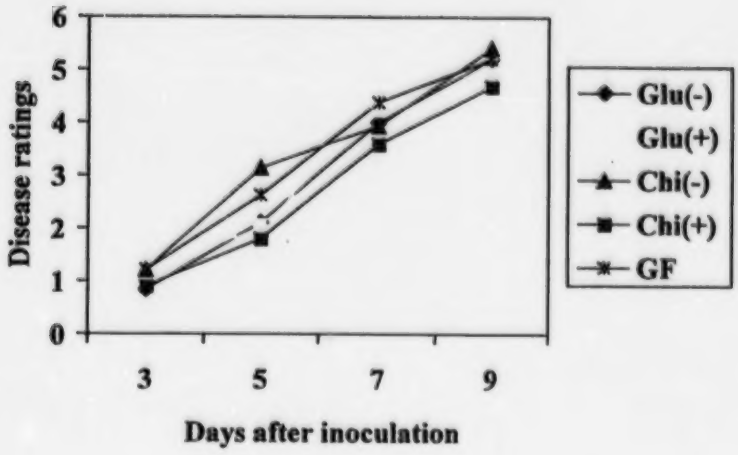


Figure 10. Effect of glucanase or chitinase gene expression on disease symptoms of detached leaves following infection with *M. pinodes* spores. Disease index used in this study is detailed Table 6. GF, untransformed Greenfeast leaves; Glu+, Greenfeast transgenics expressing the prbcS-glucanase from pFL500;Glu-, transgenics in which glucanase expression was undetectable; Chi+, transgenics expressing the CaMV35S-chitinase from pFL491; Chi-, transgenics in which chitinase expression was undetectable. Data represent the average of samples from 6 T_2 plants.

the rbcS glucanase fusion. However, leaves from plants expressing the CaMV 35S chitinase gene consistently showed reduced disease symptoms.

(ii) Plants containing a combination of antifungal protein genes.

Results from detached leaf assays using this material is presented in Figure 11. In these assays, we consistently observed that plants expressing the combination of CaMV 35S glucanase and CaMV 35S chitinase displayed less disease symptoms than the untransformed controls, than transgenic plants that did not express these genes and than plants that expressed only the CaMV 35S chitinase gene. On average, the reduction of disease symptoms observed was equivalent to 1 to 1.5 units of the disease index. The reduction of disease symptoms was observed from as early as 3 days post-infection and lasted until the last time point observed at 11 days post-infection.

Leaves from plants expressing the combination of rbcS defensin and CaMV 35S chitinase did not appear to behave differently from the plants that expressed only the CaMV 35S chitinase gene. Leaves from both these populations displayed reduced disease symptoms relative to the non-transformed plants and those that did not express transgenes. However, the reduction of disease symptoms was not as great as observed with the glucanase/chitinase combination.

DISCUSSION OF RESULTS AND CONCLUSIONS

We were successful at producing transgenic pea lines expressing high levels three different antifungal protein genes, alone or in combinations. For two of these genes, glucanase and chitinase, we demonstrated that transgenic plants possessed higher levels of corresponding enzymatic activity than untransformed pea plants.

Importantly, we were able to demonstrate that transgenic pea lines expressing chitinase or defensins alone showed reduce disease symptoms following infection with *M. pinodes* in a detached leaf assay. A line containing a combination of glucanase and chitinase was found to be the most effective material at reducing disease symptom, suggesting an additive or perhaps even synergistic effect between these two antifungal protein genes. Such effects have previously been reported in the literature (Jach et al., 1995; Zhu et al., 1994).

Although our results are encouraging, it is too early to predict the commercial value of the enhanced tolerance imparted by expression of the antifungal protein genes. In no cases did expression of the antifungal protein genes confer true "resistance" or "immunity" to infection. This was not surprising, since immunity has never been observed in other plant species overexpressing antifungal protein genes (Zhu et al., 1994; Jach et al., 1995; Terras et al., 1995; Lorito et al., 1998). We have attempted to compare the level of resistance that we observed in peas with those in the published literature. However, this was not straightforward, because methods of assessing disease symptom vary considerably between studies.

The 1 to 1.5 point reduction in disease index that we observed in plants expressing chitinase and glucanase may not appear to be dramatic at first glance. However, depending on when after infection the leaves were observed, this reduction can indicate a reduction of 50% in the necrotic area, and the difference between mycelial growth being contained within, or spreading beyond the site of infection. Such reductions in the spread of fungal growth may be very significant under field conditions. Clearly, the commercial value of peas expressing antifungal proteins will require additional testing, as described in the next section.

In addition to demonstrating the potential usefulness of transgenic approaches for reducing fungal disease, we also obtained important information about the effectiveness of

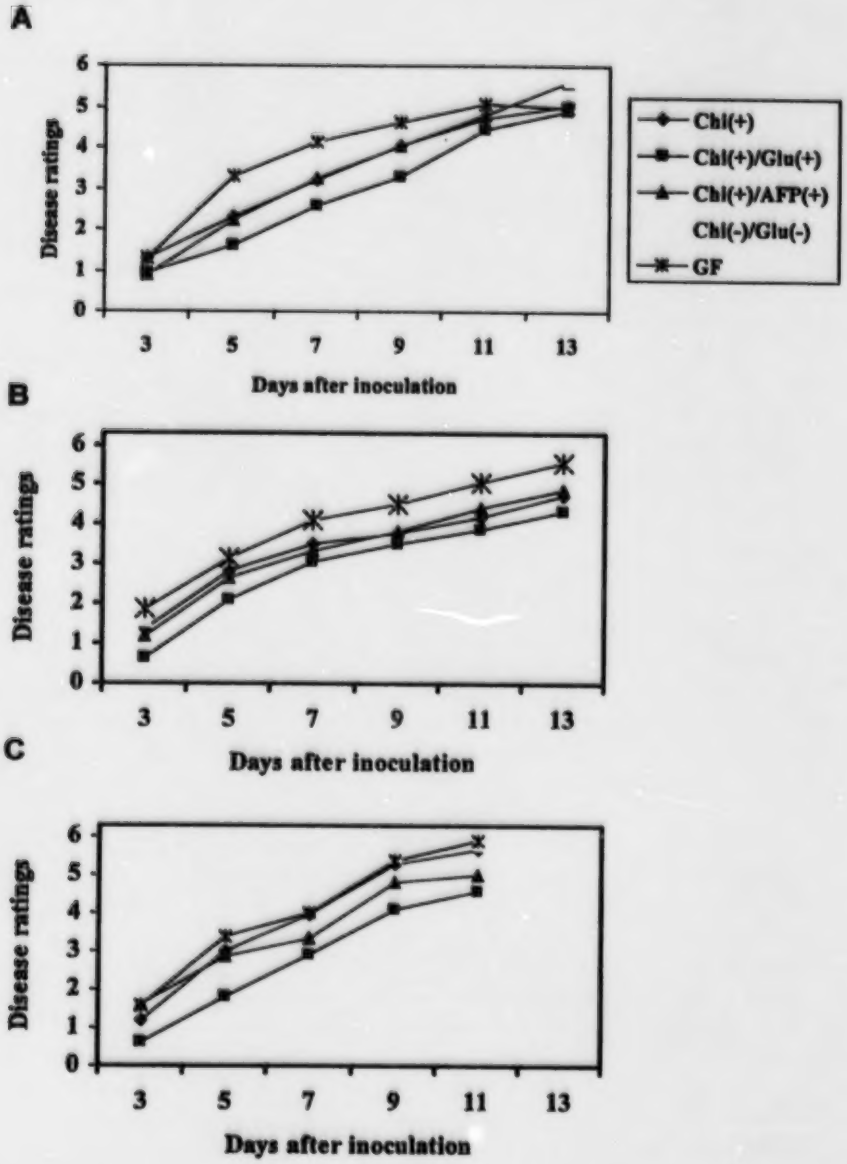


Figure 11. Effect of glucanase and chitinase combination on disease symptoms of detached leaves following infection with *M. pinodes* spores. Disease index used in this study is detailed Table 6. GF, untransformed Greenfeast leaves; Chi+, Greenfeast transgenics expressing the CaMV35S-chitinase from pFL491;Chi+/Glu+, transgenics expressing both chitinase and a CaMV35S-glucanase from pFL460; Chi+/AFP+, transgenics expressing both chitinase and a rbcS-defensin from pFL622; Chi-/Glu-, transgenics in which expression of neither chitinase nor glucanase was detectable. Data represent the average of samples from 6 plants repeated at three different times (A, B, C).

different transformation vectors with peas. In particular, our data suggest that minor changes in the orientation and/or positioning of genes within the T-DNA can greatly affect performance, and that the presence of multiple CaMV 35S promoters within the same T-DNA can have deleterious effects on transgene expression. These findings have important implications for the design of future transformation vectors. The research has also generated several hundred transgenic pea lines containing various antifungal protein genes and combinations of these genes. This collection of material represents an important resource that can be exploited in future studies of disease resistance in peas. The transformation vectors that we produced may also be useful for the transfer of antifungal protein genes to other pulses, such as lentils or chickpea.

POSSIBILITIES FOR FUTURE RESEARCH

A better indication of the effectiveness of the transgenic lines may be obtained by testing for response to *M. pinodes* infection at the whole plant level. We have initiated discussions with both Dr. Sabine Banniza (U of S, CDC) and Dr. Allen Xue at AAFC Morden, with the goal of pursuing studies in growth cabinets over the winter months. These activities may be initiated as soon as January 2000. Should results look promising, the next logical step would be to test the material in confined field trial in disease nurseries. In parallel, we would still need to generate true breeding material from the lines expressing defensin, glucanase, and those containing combinations of antifungal protein genes. Should the results from whole-plant testing look promising, it would be highly desirable to introduce the genes into elite germplasm for Saskatchewan growers.

Given the current public concerns about the safety of biotech products, especially in the traditional European legume markets, we do not foresee the possibility of commercialization of the material at any time soon. However, we believe that it is very important to continue to maintain the infrastructure for applying biotechnology to legume problems. Results obtained in the current study are a contribution towards this goal.

(c) Personnel:

The project funded two full-time personnel, each spent 100% of their time (37.5 hr/week) working on the project. Dr. Fang-Ming Lai was hired as a post-doctoral research associate (RA) from October 8, 1996 until October 31, 1999. Ms. Kangfeng Mei was hired as a technical officer (TO) from September 15, 1997 until March 31, 1999. At that time, Ms. Mei decided to leave the project for a longer-term contract. The salaries paid to these employees are detailed in the expense statement (h).

(d) Equipment

Funds provided by Sask ADF were used to buy consumables, such as molecular biology reagents, hybridization membranes, plant cell and tissue culture reagents, as well as soil and pots in which to grow plants. No equipment was purchased with the funds.

(e) Project Developed Material

A list of abstracts presented at scientific conferences and workshops are appended to the end of this report. A copy of a poster presented at the 7th International Congress of Plant Pathology, Edinburgh, Scotland, 9-16 August, 1998 is also attached.

(f) Project Photos

There are no project photos of note to include.

(g) Acknowledgements

Financial support from the Saskatchewan Agricultural Development Fund was acknowledged in the following presentations or publications:

1. Enhancing fungal disease resistance in peas through genetic engineering. PBI Seminar presented by Dr. Lai, Feb. 17, 1999
2. LAI, F.-M., K. MEI, and P. FOBERT. 1998. Genetically engineering peas with elevated levels of antifungal proteins as a means of enhancing fungal disease resistance. An oral presentation by Dr. Lai at the Pulse Crop Research Workshop, Delta Bessborough, November 27-28, 1998.
3. LAI F.-M., K. MEI, C. DELONG AND P. FOBERT. 1998. Analysis of the possible involvement of pea defensins in disease resistance. A poster presentation by Dr. Lai at the 7th International Congress of Plant Pathology, Edinburgh, Scotland, 9-16 August, 1998.
4. PBI 50th Anniversary Open House: Financial support from ADF acknowledged on two posters presented by the Legume Biotech Group. October, 1998 (posters are still on the wall at PBI)
5. Biotech Approaches for Enhancing Disease Resistance in Peas. A seminar presented by Dr. Fobert at the Agriculture and Agri-Food Canada, Morden Research Station, Morden, Manitoba. June 26, 1998.
6. FOBERT, P., F.-M. LAI, K. MEI, AND C. DELONG. 1998. Manipulating Pathogen-Related Proteins – One, a Few, or Several at a Time. Invited oral presentation by Dr. Fobert at the Joint Annual Meeting of the Canadian Society of Plant Molecular Biology, Canadian Genetics Society and Canadian Federation of Biological Sciences. Edmonton, Alberta, June 16-19.
7. Financial support of Sask ADF acknowledged during seminar by Dr. Lai during a 2-day mini-symposium of research activities at PBI, May 1998.
8. FOBERT, P., F.-M. LAI, K. MEI, AND C. DELONG. 1998. Genetically engineering peas for enhanced resistance to fungal pathogens. Invited Oral Presentation by Dr. Fobert at the 5th International Workshop on Pathogenesis-Related Proteins in Plants: Signaling Pathways and Biological Activities. Aussois, France. March 29-April 2.
9. FOBERT P.R. AND F.-M. LAI. 1998 Genetically engineering peas for enhanced resistance to fungal pathogens. Abstract for the Pulse Crop Research: Progress Reports on Pulse Crops Research in Western Canada. Volume 2.
10. FOBERT, P., DELONG, C., M. MARKO and F.-M. LAI. Anti-Fungal Proteins an oral presentation by Dr. Fobert at the Agriculture and Agri-Food Canada Workshop on Pulse Crop Research, Calgary, Alberta, November 29, 1996.

(h) Expense Statement:

	Allotment	Expenditure	Commitment	Free Balance
Salary – RA	$ 155,000.00	$ 179,381.12	–	$ (24,381.12)
Salary – TO	$ 67,000.00	$ 59,174.02	–	$ 7,825.98
Supplies	$ 33,000.00	$ 22,222.42	–	$ 10,777.58
Travel	$ 9,000.00	$ 5,725.80	–	$ 3,274.20
Audit	$ 3,000.00	–	–	$ 3,000.00
Total	**$ 267,000.00**	**$ 266,503.36**	**–**	**$ 496.64**

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Appendix B. Project Development Material – Copies of Abstracts and Posters

(Pulse Crop Research Workshop, Delta Bessborough, November 27-28, 1998)

Genetically Engineered Peas with Elevated Levels of Antifungal Proteins as a Means of Enhancing Fungal Disease Resistance

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In western Canada, fungal diseases cause a great deal of loss in field pea production. Due to lack of natural resistance to important fungal pathogens such as *Mycosphaerella pinodes*, conventional breeding faces difficulties in terms of improving fungal resistance. In our group, genetic engineering of peas is being undertaken with the aim of increasing levels of proteins capable of inhibiting fungal growth (i.e. antifungal proteins) and hence fungal disease resistance. The three classes of antifungal protein genes that we chose to transfer into peas are effective in reducing fungal disease symptoms when expressed at high levels in other transgenic plant systems. This is especially true when at least two of antifungal protein genes are co-integrated into a plant genome, where synergetic effects of antifungal activities are observed.

Transformation vectors contained the GUS reporter gene, a selectable marker gene conferring resistance to the antibiotic kanamycin, and one of the antifungal protein genes, namely β-1,3-glucanase from alfalfa, chitinase from *Trichoderma harzianum* and defensins from either pea or radish. A variety of vectors were created to test the effects of different orientations of transgenes in reference to the left or right borders of the T-DNA and in reference to the selection marker/reporter genes. The antifungal protein genes were either fused to a strong constitutive promoter (i.e. the cauliflower mosaic virus double 35S promoter with a viral translational enhancer – aka CaMV 35S) or a light-inducible promoter (i.e. the one from ribulose bis-phosphate carboxylase/oxygenase small subunit – aka RUBISCO). Agrobacteria-mediated transformation procedures were conducted using sliced embryonic axis from the cultivar "Greenfeast" as explants. The transformation vectors were initially tested in tobacco plants before pea transformation, due to the ease of the tobacco transformation.

<u>Testing the Antifungal Activity of Pea Defensins:</u>

Because the pea defensins had not been previously analyzed with respect to antifungal activity, extracts from transgenic tobacco plants were prepared, and their effect on fungal growth inhibition was tested by *in vitro* tests. Preparations from tobacco plants expressing either of two pea defensin genes, PI39 and PI230, exhibited antifungal activities. In microplate titre assay, microscopic observation, detached leaf and fungal culture analysis, co-incubation of fungal spores with defensin preparations resulted in reductions in viable number of fungal spores, germination of spore and mycelium growth. After 18 hours of co-incubation of fungal spores with defensin preparations, the disease symptoms induced by pathogenic fungal spores on detached leaves were reduced as compared to treatment with protein preparation from control, non-transgenic tobacco plants.

Production and Analysis of Transgenic Peas:

So far we obtained about 120 independent putative transgenic pea plants with at least 10 transformants from each antifungal protein gene. For some unknown reasons, only a small portion of transgenic plants (5-10 %) expressed high levels of the transgenes when driven by the CaMV 35S promoter. Other plants contained the transgenes, but did not express them. In contrast, most of the plants (ca. 90%) expressed transgenes under the control of the RUBISCO promoter, but levels of expression from this promoter were never as high as those obtained with the CaMV 35S promoter. Using PCR, Southern (DNA) and Northern (RNA) blot analyses, several transgenic lines expressing high levels of antifungal protein genes were identified. These plants were morphologically normal, and fertile. Seeds harvested from the T_0 plants were planted in soil.
The changes in fungal disease resistance are being performed on these T_1 plants. Transgenic plants containing glucanase and chitinase genes will then be crossed to produce F_1 generation plants containing both genes. Further molecular screening will be required to identify plants containing both glucanase and chitinase genes, and to test the possible synergetic changes in fungal disease resistance.

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5.3.15

ANALYSIS OF THE POSSIBLE INVOLVEMENT OF PEA DEFENSINS IN DISEASE RESISTANCE

F-M LAI , K MEI, C DELONG and P FOBERT

Background and objectives

Mycosphaerella pinodes is a serious fungal pathogen of peas in the Canadian prairies, for which there is currently no effective source of natural resistance. As a possible means of enhancing resistance to this fungus, we are producing transgenic plants expressing high levels of antifungal molecules. One class of molecules being evaluated is the defensins - small, cationic, antimicrobial peptides present in a variety of organisms. In peas, two cDNA clones isolated following fungal challenge, PI39 and PI230 [1], encode peptides that share sequence homology to known defensins. The objective of this study was to determine the possible role of these genes in disease resistance and to evaluate their potential effectiveness in enhancing tolerance to fungal pathogens in transgenic plants.

Results and conclusions

To test whether the proteins encoded by PI39 and PI230 possess antifungal activity, their coding regions were fused separately to the strong, constitutive CaMV 35S promoter and used to produce transgenic tobacco plants. Compared to peptide preparations from untransformed plants, those from plants expressing high levels of pea defensin transcripts were considerably more effective at reducing growth of the test fungus *Trichoderma reesei* in a microtitre plate bioassay. These preparations appeared to be less effective at inhibiting the growth of *M. pinodes*. In contrast, peptide preparations from transgenic tobacco plants expressing the radish defensin RsAFP2 appeared to be equally effective at inhibiting growth of *T. reesei* and *M. pinodes*. We are currently testing a range of fungi to determine if *M. pinodes* may be more tolerant to the pea defensins.

We also examined the expression pattern of the pea defensins in detail. Transcripts of both genes were detected at high levels in numerous tissues from unchallenged plants, including mature leaflets, tendrils, stems, flowers and radicles from axenically germinated seedlings. Immature seed pods, leaflets from seedlings and mature roots expressed PI39 and PI230 at either lower or non-detectable levels. Following challenge of mature leaflets with compatible, incompatible and non-host strains of *Pseudomonas syringae*, moderate increases in transcript levels were observed for both genes. In comparison, levels of a PR10 homologue were barely detectable prior to challenge, and increased dramatically thereafter. Overall, the expression pattern of PI39 and PI230 suggests that they may represent preformed resistance factors, or possibly be involved in non-defence-related processes.

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Analysis of the possible involvement of pea defensins in disease resistance

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Introduction

Defensins are small, cationic anti-microbial peptides that have been identified in many different organisms. In peas, two cDNA clones, namely PI39 and PI230, isolated after fungal pathogen challenge (1), encode peptides that share sequence homology to other known plant defensins (Figure 1).

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Pea     PI39   -NT -ENLADT K V YTHAS DDN KNK H-LIS K N-DW---R W TKN
Pea     PI230  -NT -ENLAGS K V YGG-- DRH RTQ G-AIS R RDDF---R W TKN
Radish  AFP1   IKL -ERPSGT S V GNNNA KNQ INL X-ARN S NYVFPAHK I YFP
Radish  AFP2   IKL -QRPSGT S V GNNNA KNQ IRL X-ARN S NYVFPAHK I YFP
CONSENSUS      --  ------ - - ----- --- --- ----- - --------- - ---
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Figure 1
Peptides encoded by PI39 and PI230 share structural consensus with other plant defensins.

In this study, the role of PI39 and PI230 in terms of anti-microbial activities was analyzed by (a) monitoring their expression in different tissues; (b) comparing their expression with other known PR gene expression following challenge with bacterial and fungal pathogens; and (c) using crude peptide preparations from transgenic tobacco plants expressing PI39, PI230 and radish defensin genes to test the anti-fungal activities.

Materials and Methods

Northern analysis

Total RNA (5 µg), isolated from plant tissues using Trizol reagent, was separated on a 1.2% agarose gel containing 1.5% formaldehyde, blotted onto a nylon membrane (GeneScreen Plus, NEN Life Science Products) and hybridized with α-^{32}P-labeled probes.

Crude peptide preparation

Total soluble protein was extracted from leaf tissue of either untransformed or transgenic tobacco plants expressing PI39, PI230 or radish AFP2 genes under the control of the CaMV-35S promoter. The extraction buffer contained 50 mM Tris, 1 mM PMSF, 2 mM thiourea and 1.5% PVP, pH 7.5.

After heat-treatment at 85°C for 10 min, the denatured proteins were removed by centrifugation. The remaining soluble proteins were precipitated by adding ammonium sulfate to 85% saturation, solubilized into water, and dialysed against 50 mM Tris buffer, pH 7.5. This solution was concentrated with a Centricon-3 spin column to obtain the crude peptide preparation.

Microtitre antifungal test

Fungal spores (2000 spores/well) were incubated with peptide preparations at room temperature in 1/8 strength PDB (potato dextrose broth) for 2 to 5 days. Fungal spore germination and growth were monitored with a light microscope or a microtitre plate reader according to Broekaert *et al.* (2).

Summary

a) PI39 and PI230 genes are expressed at high level in unchallenged pea plants. Expression of the two pea defensin genes is developmentally regulated with higher expression in the unchallenged mature tissues and no expression in the young pod tissues.

b) The expression of the two pea defensin genes is enhanced following challenge with fungi, similar to that of the other known PR genes. Similar results were observed following challenge with bacteria pathogens (e.g. *Pseudomonas syringae*) (data not shown).

c) Pea defensins prepared from transgenic tobacco plants have antifungal activities against a broad range of fungi tested in vitro.

Conclusion

The expression pattern and antifungal activity of PI39 and PI230 indicate that these peptides could play a role in preventing or limiting microbial infection, possibly as pre-formed, as well as pathogen-inducible factors.

Acknowledgement

We would like to thank the Saskatchewan Agricultural Development Fund, Canada for financial support.

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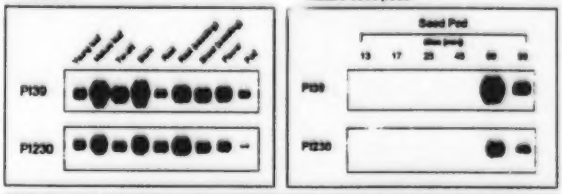
Results

Pea defensin gene expression is developmentally regulated

a) PI39 and PI230 expression in different tissues.

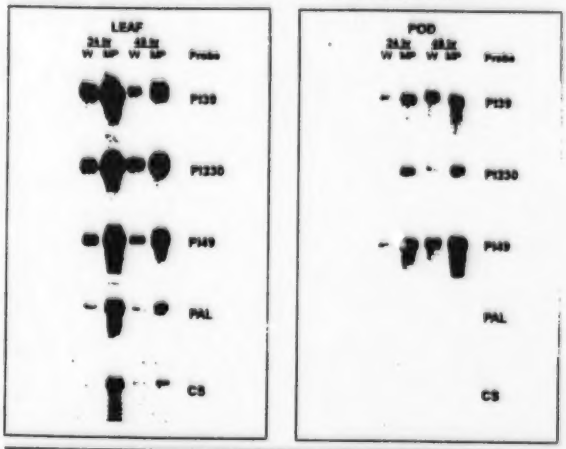
b) PI39 and PI230 express only in more mature seed pods.



Pea defensin gene expression is enhanced following challenge with a fungal pathogen

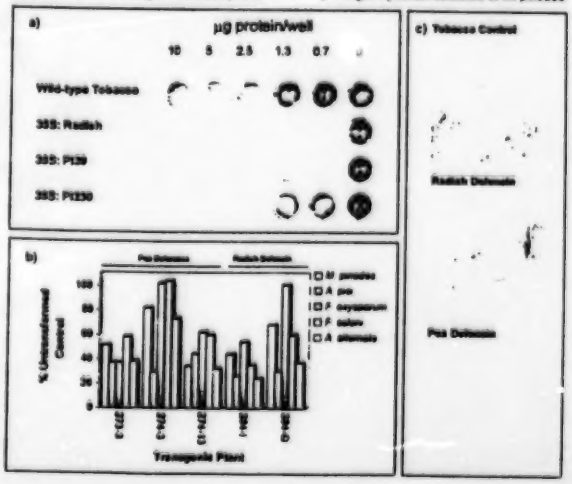
a) PI39 and PI230 expression in leaves following challenge with water (W) or *Mycosphaerella pinodes* (MP), in comparison with PI49 (a PR10 homologue), chalcone synthase (CS) and PAL gene expression.

b) PI39 and PI230 expression in pods following challenge with water (W) or *Mycosphaerella pinodes* (MP), in comparison with PI49, chalcone synthase (CS) and PAL gene expression.



Antifungal activity of pea defensins: Antifungal test in microtitre plates using crude defensin preparations from transgenic tobacco plants.

a) Microtitre plate assay
b) Growth inhibition
c) Fungal mycelium structure of *M. pinodes*



Abstract for: 1998 Joint Meeting of the Canadian Society of Plant Molecular Biology, CFBS and the Genetics Society of Canada. June 17-19, Edmonton, Alberta.

Manipulating Pathogen-Related Genes – One, a Few, or Several at a Time.

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A popular biotech strategy for enhancing resistance to fungal pathogens consists of over-expressing one or a few molecules having antifungal properties in transgenic hosts. With the recently acquired ability to produce transgenic peas, we have undertaken a project to introduce different antifungal genes into this crop. In parallel, we have been characterizing pathogen-related (PR) genes from peas, to assess their potential as targets for future over-expression studies. In an attempt to coordinately increase the expression of a large number of PR genes, we are studying the signal transduction pathway leading to their expression. Our work is focused on the *npr1* gene of *Arabidopsis thaliana*, which is required for PR gene expression leading to systemic acquired resistance. Using the yeast-two hybrid system, proteins capable of interacting the NPR1 have been identified. Possible mechanisms of action for NPR1 will be discussed based on these findings.

APPROACHES FOR GENETICALLY ENGINEERING PEAS FOR ENHANCED RESISTANCE TO FUNGAL PATHOGENS

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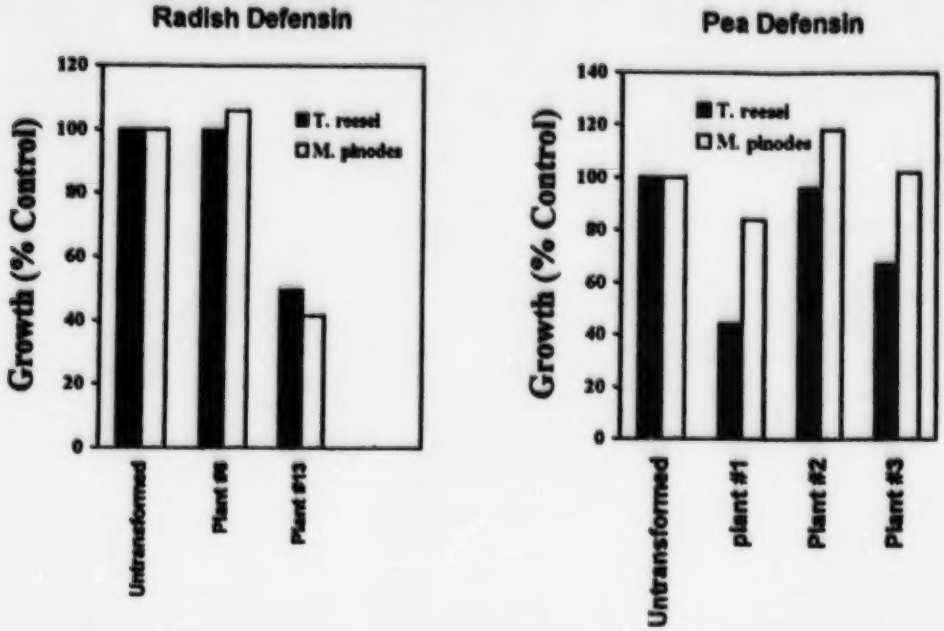
In an effort to enhance resistance of peas to fungal pathogens such as *Mycosphaerella pinodes*, for which there remains no effective source of natural resistance, plants are being genetically engineered to express high levels of proteins capable of suppressing fungal growth. The proteins being evaluated act either by forming "holes" in the fungal cell membrane (e.g. defensins) or by breaking-down the major carbohydrates in the fungal cell wall (e.g. β-1,3 glucanase and chitinase). Several transformation vectors containing the protein-coding portion of the antifungal genes fused to regulatory elements capable of driving high levels of expression (CaMV 35S or rbcS promoters) were synthesized and transferred into tobacco for preliminary testing. (Tobacco transformation is much more rapid and efficient than pea transformation.)

Extracts from transgenic tobacco expressing defensins were screened to identify those most effective at inhibiting fungal spore germination and growth in a microtititre plate bioassay. When compared to extracts from untransformed tobacco plants, those from transgenic plants expressing a defensin from radish (RsAFP2) or two putative defensins from peas were considerably more effective at reducing growth of the test fungus *Trichoderma reesei* (see Figure). However, the radish defensin appeared to be more effective at inhibiting growth of *M. pinodes* than either of the pea defensins.

We are currently testing a range of fungi to determine whether the greater tolerance of *M. pinodes* to the pea defensins is a general characteristic of pea pathogens. The results also prompted us to study the expression of these genes prior to, and following pathogen challenge. Our results indicate that pathogen attack is not required to induce relatively high levels of expression in stems, mature leaves, flowers and pods. In fact, gene transcripts were detected even when the plants were grown under sterile conditions. Pathogen attack led to a modest increase in transcript levels. For comparison, we studied the expression of other pathogenesis-related genes, such as glucanase, PR10 and PI206, and found these to be expressed at high levels only following pathogen attack.

Based on the above observations, we have concentrated on producing transgenic pea plants containing the radish defensin gene. To date, in excess of 4,000 explants have been infected with *Agrobacterium* strains containing this gene fused to the CaMV promoter. This has yielded at least 15 putative transgenic shoots (conclusive demonstration of transformation is in progress), 2 of which have flowered and set seeds. These plants are anatomically normal and indistinguishable from untransformed plants. They also express a marker gene (GUS) present in the transformation vector, which suggests that they are, in fact, transgenic. Seeds from the plants are being sown and progeny will be analyzed for the presence and segregation of the radish defensin gene, level of expression, and antifungal activity. Because of the long time-

frame associated with pea transformation, several additional plants are likely to be produced from the material we now have in culture.



Work with β-1,3 glucanase and chitinase genes has focused on the production of effective transformation vectors. For reasons we do not completely understand, the orientation and/or position of these antifungal genes relative to our selectable marker gene (used to select rare transformed cells among large populations of untransformed cells), has a critical influence on our ability to recover transgenic plants. This is true in both tobacco and peas. However, we have now identified effective transformation vectors, and *Agrobacterium* strains containing these vectors have been used to infect in excess of 2,000 pea explants. This material is still being grown in tissue culture, and the first wave of putative transgenic shoots are beginning to appear.

Our efforts to transform peas will continue over the winter months. Because foreign genes can be expressed at variable levels, or succumb to structural re-arrangements in transgenic plants, our goal is to produce at least 10 independent transformed plants expressing each of the antifungal protein genes, and choose the best performers for further testing. In other plant species, it has been suggested that combinations of specific antifungal proteins can interact synergistically to reduce disease incidence. Therefore, one priority will be to cross transgenic pea plants expressing the various classes of antifungal proteins in an effort to further enhance resistance to *M. pinodes*.

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ANTI-FUNGAL PROTEINS. Pierre R. Fobert, Catherine Delong, Melanie Marko and Fang-Ming Lai. Legume Biotechnology Group, Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, S7N 0W9.

Although it is generally accepted that genetic resistance is the "most economical, efficient, environmentally acceptable and long-term sustainable" means of disease control[1], the fact remains that effective sources of natural resistance are not available for some plant-pathogen systems. This appears to be the case for resistance to *Mycosphaerella pinodes* in peas, where extensive searches have failed to identify sources of resistant germplasm. However, the ability to genetically transform peas (see abstract by Polowick) offers the potential to introduce into the genome of these plants an unlimited source of new genetic information, including genes that may enhance resistance to fungal pathogens. One such class of genes are those which encode proteins capable of directly inhibiting fungal growth. These antifungal proteins are naturally produced by plants in response to pathogen attack, but fail to accumulate fast enough or at high enough levels in susceptible plants because virulent pathogens can often suppress plant defense responses or escape their detection. Therefore, one possible strategy of enhancing resistance to pathogens, including *M. pinodes*, would be to genetically engineer plants that contain high levels of key antifungal proteins prior to pathogen attack, instead of following it. This would ensure the presence of high-levels of antifungal proteins from the earliest stages of attack, a time during which susceptible plants fail to produce substantial amounts of defense proteins. There is good evidence from work in canola and tobacco that such a strategy can be used to enhance resistance to fungal pathogens in the lab as well as the field[2].

Our initial focus has been on a class of small cationic antifungal peptides known as defensins[3]. Defensins are thought to integrate into cell membranes to create ion channels, leading to eventual cell death. They are generally active against a broad range of micro-organisms, including bacteria and fungi, although some are more active against fungi. However, defensins are not active against plant and animal cells. We have isolated the coding regions of a radish defensin gene known to have antifungal properties[4] and two putative defensin genes from peas[5]. Each of these coding regions was fused to regulatory elements (promoter) that allow their expression at high levels throughout uninfected plants, and transferred to the *Agrobacterium tumefaciens* strain EHA105 as a prelude to plant transformation. Because pea transformation is still slow and labour-intensive, we transferred the defensin genes into tobacco plants for preliminary testing. Several transgenic tobacco plants containing the pea defensins have been produced and are now being tested for enhanced antifungal activity against a range of pathogens. These tests will allow us to confirm whether the gene constructs function in plants and may tell us whether any of the defensins is more effective at inhibiting growth of important pea and/or lentil pathogens. Should results look promising, we will begin to transfer the genes into pea plants this winter.

In addition to defensins, we plan on producing transgenic pea plants which contain high levels of other antifungal proteins such as chitinase and ß-1,3-glucanase, which degrade the main carbohydrate components of fungal cell walls, chitin and ß-1,3-glucan, thereby inhibiting fungal growth. Because different classes of antifungal proteins may interact to achieve higher levels of fungal growth inhibition[2], we will produce transgenic pea plants containing combinations of defensin, chitinase and/or ß-1,3-glucanase in an attempt to further enhance resistance to fungal pathogens. An added benefit of this strategy is that it may also provide a more durable resistance since it should prove more difficult for pathogens to overcome multiple, instead of single, antifungal proteins.

ACKNOWLEDGMENTS: This work is supported by a grant from the Saskatchewan Agricultural Development Fund.

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